The Significance of Aryl Hydrocarbon Hydroxylase Enzyme Systems in the Selection of Model Systems for Respiratory Carcinogenesis*

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ABSTRACT

Aryl hydrocarbon hydroxylase (AHH) is a multicomponent, microsomal-bound enzyme system which converts a variety of lipid-soluble compounds to water-soluble forms for subsequent elimination from the body. The enzyme system is inducible by a variety of endogenous and exogenous compounds including steroid hormones, barbiturates, insecticides, polycyclic aromatic hydrocarbons (PAH), and whole cigarette smoke. Recent results have demonstrated that inducibility is host-gene regulated, the inducibility of this enzyme correlates with carcinogenic susceptibility to PAH in animal, and bronchogenic squamous cell carcinoma in humans (probably cigarette smoke induced).

This paper illustrates the types of AHH responses observed in pulmonary tissues following treatment of mice of various strains with either PAH or tobacco related chemicals. Following intratracheal instillation of 3-methylcholanthrene, we observed that: a) pulmonary AHH can be induced preferentially at doses <200 µg (in contrast to higher doses that induce both hepatic and pulmonary tissues), b) kinetic data demonstrate a 6 to 8 fold increase within 24 hours followed by a broad plateau lasting up to 96 hours, and c) induction is host regulated, segregating as a single autosomal dominant gene in crosses between the C57BL/6 (inducible) and DBA/2 (noninducible) strains of mice. Although DBA/2 pulmonary tissue is slightly inducible (in contrast to the noninducibility of hepatic tissue), evidence indicates that this response results from proliferation of constitutive AHH and not true "induction". Exposure to whole smoke from one 1A1 cigarette (10% smoke in a Walton Horizontal Smoking Machine) will preferentially induce pulmonary AHH, and this response is under the same genetic control as that induced by MCA. Exposure to gas phase alone will not induce this response. Use of cigarette smoke condensate fractions (Stedman fractionation) derived from 1A1 tobacco show that after intratracheal instillation, at least 4 fractions are capable of inducing pulmonary AHH. Fractions 3, 4, 12, and 14 (Bi^a, Bi^b, N_{NM}, and N_{MEOH}) induce at least a 2 fold increase of pulmonary AHH (at a LD_{10-14} dose). It seems as if the enzymatic potential of the lung tissue itself may be a major determinant in the ultimate fate of this organ in any carcinogenic process.

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A. Introduction

Aryl hydrocarbon hydroxylase (AHH) is the name given to one of the multi-component, mixed-function oxidases that converts a variety of lipid-soluble endogenous and exogenous compounds to water-soluble forms, usually for subsequent elimination from the body (MASON, 1957; CONNEY, 1967). The enzyme system possesses 2 properties which make it particularly amenable for studying its role in chemically induced cancers: 1. the system is inducible**(NEBERT and GELBOIN, 1969) and 2. this inducibility is regulated by a single autosomal dominant gene in crosses involving the C57BL/6 and DBA/2 strains of mice (THOMAS et al., 1972; NEBERT et al., 1972). Recent information suggests that this enzyme system plays a major role in 3-methylcholanthrene-induced carcinogenesis in the aforementioned mouse strains (KOURI et al., 1973a, 1973b, 1974a).

The lung and skin of mice seem to be under a different type of genetic control from that of hepatic tissue, because these organs appear to be slightly inducible in strains in which the liver is completely non-inducible (BURKI et al., 1973; WIEBEL et al., 1973). There are many questions concerning the pulmonary response: Is there really a separate genetic control for lung AHH levels? Do genetically regulated differences in AHH inducibility exist in pulmonary tissue of inbred mice? What is the effect of other exogenous chemicals (e.g., tobacco-related products) on lung AHH? Do these enzymatic responses play a role in the susceptibility of mice to chemically induced lung cancers? In this report, we attempt to answer some of the questions concerning the pulmonary response.

B. Materials

The polycyclic hydrocarbons benzo(a)pyrene (BaP) and 3-methylcholanthrene (MCA) were purchased (Sigma Chemicals, St. Louis, Missouri) and purified by recrystallization from benzene. 7,8-benzoflavone was purchased from Aldrich Chemicals (Cedar Knolls, New Jersey). Sources of mice were Cumberland View Farms (Clinton, Tennessee), the Jackson Laboratory (Bar Harbor, Maine), or Microbiological Associates (Bethesda, Maryland). For intratracheal (IT) instillations, a Bausch and Lomb stereomicroscope, equipped with fiber optic illumination, and Hamilton syringes with 22 gauge by 38 mm (with 1.5 mm feeder balls) needles were used. Fractions of cigarette smoke condensate from 1A1 cigarettes were provided by Dr. A.R. PATEL (Meloy Laboratories, Springfield, Virginia)(PATEL et al., 1974). Fractionation into acidic, basic, and neutral fractions was performed according to the procedures of SWAIN et al. (1969). Only 3 fractions have been analyzed as to chemical content: the B_E fraction contains 310 mg nicotine per g fraction, the WA_E fraction contains 41.9 mg phenols per g fraction, and the N_{NM} fraction contains 13.2 µg BP per g fraction (PATEL et al., 1974). Walton-type horizontal smoking machines were obtained from Process and Instruments (Brooklyn, New York), cigarettes were either the 1A1 or 1R1 type (University of Kentucky, Lexington). Enzyme determinations were made

** The term "inducibility", as used in this paper, denotes a relative increase in rates of de novo synthesis or of activation of enzyme activity from preexisting moieties, or in rate of both when compared to rate of breakdown. No particular mechanism is implied.

using an Aminco-Bowman spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland).

C. Methods

Care and feeding of mice were as previously published (WHITMIRE et al., 1971). Animals were always treated between the hours of 9:00 am and 10:00 am to avoid diurnal variations. The intratracheal instillation technique was similar to that described recently by HO and FURST (1973). Solutions consisted of MCA suspended in 0.2% gelatin in sterile saline or the various cigarette smoke condensate fractions dissolved in corn oil. Condensate fractions were used at an arbitrary level that killed 10% of the mice in 14 days (LD_{10-14}). 0.02 ml of solution was instilled. At various times post-treatment, lungs and livers were excized and frozen at -70°C until assayed.

Microsomes were prepared from liver tissues of mice pretreated with MCA according to the methods of KUPFER and LEVIN (1972) and were stored at -70°C for up to 72 hours before being assayed. Calcium-aggregated and "normal" centrifugally prepared microsomes were used with similar results. Samples were diluted with 0.1M tris-HCl buffer (pH 7.4) to a final ratio of 1.0 ml microsome suspension per wet weight tissue.

The assay for AHH was basically that of NEBERT and GELBOIN (1969), as modified by NEBERT and GIELEN (1972) and THOMAS et al. (1972). A unit of AHH activity is that amount of enzyme causing the fluorescent equivalent of 1.0nMole 3-OHBP per min at 37°C. For hepatic and pulmonary tissues, activity is given in terms of units/g wet weight tissue and for microsome preparations in terms of units/mg protein.

Inhibition of BaP metabolism *in vitro* was done according to the procedures of GOUJON et al. (1972) and WIEBEL et al. (1971). Concentrations of the various condensate fractions were made in dimethylsulfoxide (DMSO); included was the known competitive inhibitor of "induced" AHH activity, 7,8-benzoflavone (WIEBEL et al., 1971). 200 µg, 20 µg, and 2 µg of the fractions were added to the complete reaction mixture (minus BaP), incubated with shaking for 1.0 min, and then BaP (20 µg) was added and incubation continued for 20 min.

Mice were exposed to 1, 2, or 3 cigarettes simultaneously (10%, 20%, or 30% smoke) and were also exposed to the smoke of 1 cigarette (10% smoke) for 1.0 hour (about 7 cigarettes). This latter exposure regimen consisted of exposing mice to 1 cigarette followed by a 10 min rest period followed by 1 cigarette, until a total of 7 cigarettes were smoked. In some cases, mice were exposed to cigarette smoke for at least 60 days at 8 cigarettes per day (4 consecutive cigarettes in morning and 4 in afternoon). At various times after exposure, mice were killed by cervical dislocation and the lungs and livers were removed and stored at -70°C until assayed. Control animals consisted of either untreated, sham-smoked, or gas-phase (Cambridge-filtered smoke) treated animals. Mice were exposed in 1 min cycles consisting of a 2 sec puff, 15 sec holding time, and 43 sec purge. In certain experiments, holding time was increased to 28 sec and purge time was 30 sec.

D. Results

I. Pulmonary and Hepatic AHH Responses to IT Instilled MCA

The pulmonary and hepatic AHH levels 24 hours after IT administration of various dose of MCA into C57BL/6Cum (B6) DBA/2Cum (D2) and B6D2F1 mice are shown in Table 1. At doses greater than 200 µg, the AHH responses of pulmonary tissues from B6 and B6D2F1 lungs were maximally induced (about 7 fold) while hepatic tissues were only minimally effected. The D2 strain was generally much less responsive: pulmonary tissue was only minimally induced at a dose of 500 µg and hepatic tissue was never induced, regardless of MCA dose. Kinetics of induction of pulmonary AHH in these 3 strains following IT treatment with 200 µg MCA are shown in Fig. 1. Maximum induction in the B6 and B6D2F1 strains occurred by 24 hours and remained constant for at least 96 hours. The D2 strain was observed to respond slowly to MCA and maximal induction occurred 48 hours posttreatment. The maximum observed increase (inducibility) for the B6 or B6D2F1 strains was about 10 and for the D2 strain about 6.

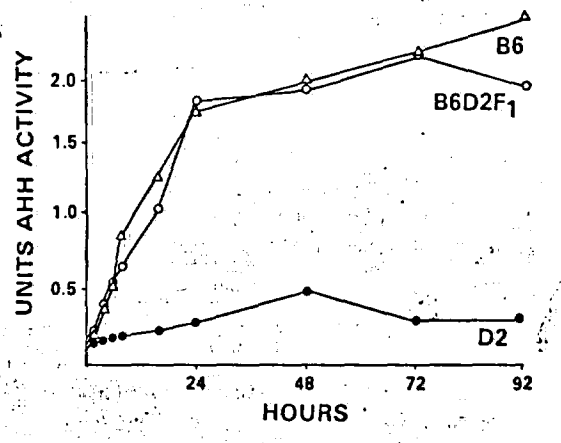

Fig. 1

Responses of 8 other inbred strains to 200 µg MCA given IT are shown in Table 2. Pulmonary tissues of BALB/cMai C3H/fMai, C57L/J, and C57BL/6J were observed to be highly induced by 200 µg MCA (4 to 8 fold), while lung tissue from strains AKR/J, SJL/J, DBA/2J, and RF/J showed no such increase. Hepatic responses were low for all strains except perhaps for the C57BL/6J and C57L/J, which did express a 1-fold increase..

II. Genetic Regulation of Pulmonary AHH Induction

The effect of MCA on pulmonary tissue from crosses involving the B6 and D2 strains is shown in Table 3. Animals were classified as inducible or noninducible if, after IT treatment with 200 µg MCA, pulmonary AHH levels were 2.5 (± 0.3) units/g tissue (inducible) or 0.3 (± 0.05) units/g tissue (noninducible). Among 47 backcross animals

Table 1. Effects of intratracheal instillation of various doses of MCA in a 0.2% gelatin solution on pulmonary and hepatic AHH[a] levels

STRAIN AND TISSUE	UNTREATED	GEL	MCA 10 µg	MCA 50 µg	MCA 200 µg	MCA 500 µg
C57BL/6Cum						
LUNG	0.40	0.32	0.89 (2.8)[b]	1.4 (4.4)	2.4 (7.5)	2.4 (7.5)
LIVER	17.50	14.50	13.30 (0.9)	14.51 (1.0)	32.3 (2.2)	33.2 (2.3)
DBA/2Cum						
LUNG	0.30	0.18	--	0.20 (1.1)	0.34 (1.9)	0.56 (3.1)
LIVER	9.80	9.50	--	9.80 (1.0)	10.10 (1.1)	10.20 (1.1)
B6D2F1Cum						
LUNG	0.36	0.26	0.76 (2.9)	1.3 (5.0)	2.0 (7.7)	2.5 (9.6)
LIVER	12.60	10.00	10.30 (1.0)	14.4 (1.4)	18.9 (1.9)	26.5 (2.7)

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole 3-OH-BP PER MINUTE AT 37°C

[b]THE INDUCIBILITY (A RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE) IS GIVEN PARENTHETICALLY

Table 2. Effects of intratracheal instillation of 200 µg MCA in 0.2% gelatin on pulmonary and hepatic AHH[a] in various strains of mice

STRAIN	LUNG AHH CONTROL	LUNG AHH MCA	LUNG AHH IND.[b]	LIVER AHH CONTROL	LIVER AHH MCA	LIVER AHH IND.
Balb/cMai	0.71	3.1	4.4	19.2	22.2	1.2
C3H/fMai	0.33	2.5	7.7	7.9	7.3	0.93
C57L/J	0.64	3.4	5.3	13.3	27.4	2.1
C57BL/6J	0.28	2.4	8.0	16.1	32.6	2.0
AKR/J	0.28	0.45	1.6	17.5	15.6	0.89
SJL/J	0.19	0.29	1.5	10.8	11.7	1.1
DBA/2J	0.26	0.36	1.4	8.9	8.5	0.95
RF/J	0.41	0.54	1.3	13.5	13.8	1.0

[a]AHH ACTIVITY GIVEN IN TERMS OF UNITS PER g WET WEIGHT TISSUES. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1.0 nmole OF 3-OH-BP PER MINUTE AT 37°C

[b]IND. = INDUCIBILITY; RELATIVE INCREASE OF AHH OF TREATED TISSUE COMPARED TO CONTROL TISSUE

tested, 24 were inducible (51%), and among 42-F2 animals tested, 29 were inducible (69%). These numbers were not statistically different from the 50% and 75% ratios that would be expected if a single autosomal dominant gene were regulating this inducibility.

Table 3. Genetic regulation of pulmonary AHH in crosses involving the C57BL/6Cum and DBA/2Cum strains of mice[a]

STRAIN	NUMBER OF MICE TREATED	NUMBER OF MICE INDUCIBLE	%
B6	50	50	100
D2	50	0	0
B6D2F1	50	50	100
B6D2F1 x D2	47	24	51
B6D2F2	42	29	69

[a]MICE WERE TREATED WITH 200 µg MCA/.02 ml 0.2% GELATIN SOLUTION INTRATRACHEALLY, AND 24 HOURS LATER, THE PULMONARY AHH WAS ASSAYED. A MOUSE WAS CONSIDERED INDUCIBLE IF, AFTER MCA TREATMENT, PULMONARY AHH WAS 2.5 (+ 0.3) UNITS/g TISSUE AND CONSIDERED NONINDUCIBLE IF PULMONARY AHH WAS 0.30 (+ 0.05) UNITS/g TISSUE. THE SEX OF THE PROGENY PLAYED NO ROLE IN THIS SEGREGATION PATTERN

III. Effect of Tobacco Related Products on Pulmonary AHH Levels

1. Effect of Cigarette Smoke. The pulmonary AHH response of B6 mice exposed to the smoke of one-1A1 cigarette (10% smoke) is shown in Table 4. The lung tissue responded rapidly and selectively to the whole smoke. Peak activity occurred approximately 6 hours posttreatment and remained induced for 24 hours. The intervention of a Cambridge-type filter completely abrogated this induction profile. The use of 2 or 3 cigarettes smoked simultaneously (20% or 30% smoke) gave induction results similar to the use of 1 cigarette (data not shown). Exposure to 1 cigarette-at-a-time for a total of 7 cigarettes (with a 10 min rest between cigarettes) induced pulmonary AHH activity in B6 and C3H/fMai mice, but had only a small effect on D2 mice (Table 5). This smoking schedule resulted in maximal exposure with minimal death if nonpretreated animals were used. Using this schedule, the maximal induction was similar to that induced by 1 cigarette (Table 4): about 2.5 fold in 6 hours. Similar results were also noted using the 1R1 cigarette, i.e., maximal induction occurred within 6 hours after exposure and induction was about 2.5 times that of the sham control or gas-phase treated animals. Similar results were also noted if the holding time was increased from 15 to 28 sec. The 28 sec holding time should allow for maximum deposition of particulate material onto lung tissue (STOCKLEY, Oak Ridge National Laboratory, personal communication, 1974).

Mice could be adapted to higher smoke exposures by pretreatment with only 1 or 2 cigarettes per day for 1 week. By slowly increasing the number of cigarettes (given 1-at-a-time) per day, at 1 month, both D2 and C3H/fMai mice would accept 16 cigarettes per day, 8 consecutive cigarettes in the morning and 8 consecutive in the afternoon. Although slightly more toxic initially, mice would still accept 16-1R1 cigarettes per day using this same schedule. The AHH responses of various tissues of these 2 strains after exposure to 8 cigarettes per day (4 in the morning and 4 in the afternoon) for at least 60 days are demonstrated in Table 6. The chronic high level of smoke seemed to induce only pulmonary tissue; liver, kidney, and small intestinal tissue was unaffected. The data with the intestines was difficult to assess because of the wide mouse-to-mouse variations observed (>200%). Pulmonary tissue from C3H/fMai mice was induced for the whole 18 hours observation period, while the induction of D2 lung tissue seemed to

Table 4. Effect of exposure to one 1A1 cigarette (10% smoke) on pulmonary AHH levels of C57BL/6Cum mice[a]

HR. AFTER SMOKE	AHH ACTIVITY (UNITS[b]/g TISSUE) WITH FILTER	WITHOUT FILTER	INDUCIBILITY
1.5	0.25	0.24	1.0
3.5	0.21	0.43	1.8
6.5	0.31	0.86	3.6
9.0	0.25	0.53	2.2
12.0	0.25	0.58	2.4
27.0	0.27	0.55	2.3
50.0	0.27	0.38	1.6
74.0	0.27	0.38	1.6
CONTROL	0.24	0.24	

[a]MICE WERE EXPOSED IN A WALTON-TYPE HORIZONTAL SMOKING MACHINE ACCORDING TO THE FOLLOWING 1 MIN CYCLE: 2 SEC PUFF, 15 SEC HOLDING TIME AND 45 SEC PURGE

[b]UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 5. Effect of 7 consecutive cigarettes[a] on pulmonary AHH activity in various strains of mice

STRAIN	CONTROL	HOURS[b] POST TREATMENT 6 SMOKED	6 INDUCIBILITY	24 SMOKED	24 INDUCIBILITY
C57BL/6Cum	0.20[c]	0.50	2.5	0.45	2.3
DBA/2J	0.25	0.34	1.4	0.32	1.4
C3H/fMai	0.34	0.88	2.6	0.65	1.9

[a]ANIMALS WERE EXPOSED TO CIGARETTE SMOKE USING THE REGIMEN OF ONE CIGARETTE FOLLOWED BY A 10 MIN REST PERIOD FOLLOWED BY ONE CIGARETTE, UNTIL A TOTAL OF SEVEN CIGARETTES WERE SMOKED

[b]HOURS AFTER EXPOSURE TO LAST OF SEVEN (7) 1A1 CIGARETTES

[c]DATA GIVEN IN TERMS OF UNITS AHH ACTIVITY PER g TISSUE. A UNIT IS THAT AMOUNT OF ENZYME CAUSING THE FORMATION OF THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP PER MIN AT 37°C

have a shorter lifetime and was at background level by 18 hours post-treatment. Responses to the 1R1 cigarettes was similar to that observed for the 1A1 cigarettes.

2. Effect of Cigarette Smoke Condensate Fractions. Fractions of 1A1 cigarette smoke condensate were observed to induce and to inhibit the pulmonary AHH activity of B6 mice 24 hours after IT instillation (Table 7). Fractions $B_I a$, $B_I b$, N_{MEOH}, and N_{NM} were considered good inducers. The starting material, reconstituted material, B_E, WA_E, WA_I, and N_{CH} were considered weak inducers. Fractions B_W, SA_I, SA_E, and SA_W were actually weak inhibitors of pulmonary AHH activity.

Particular fractions also seemed to have the capability of inhibiting BaP metabolism *in vitro* (Table 8). Data is presented by computing the

Table 6. Effect of smoking[a] on AHH responses of various tissues of 2 inbred strains of mice

STRAIN	TISSUE	HOURS POST TREATMENT 3 CONTROL	3 SMOKED	3 IND.[b]	6 CONTROL	6 SMOKED	6 IND.[b]	18 CONTROL	18 SMOKED	18 IND.[b]
C3H	LUNG	0.34	1.01	3.1	0.48	0.95	2.0	0.23	0.51	2.2
	LIVER	9.90	10.31	1.1	8.00	9.40	1.2	11.00	13.70	1.2
	KIDNEY	0.06	0.06	1.0	0.07	0.06	0.9	0.06	0.07	1.1
	INTESTINE	1.10	0.63	0.6	0.28	0.30	1.1	0.25	0.35	1.4
D2	LUNG	0.36	0.82	2.3	0.35	1.00	2.9	0.30	0.30	1.0
	LIVER	8.00	10.50	1.3	10.90	9.50	0.9	13.50	14.00	1.0
	KIDNEY	0.08	0.07	0.9	0.06	0.06	1.0	0.07	0.07	1.0
	INTESTINE	0.28	0.99	3.5	0.07	0.08	1.1	0.09	0.07	0.8

[a]ANIMALS WERE PRESMOKED FOR AT LEAST 60 DAYS BY EXPOSURE TO FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE MORNING AND FOUR (4) CONSECUTIVE CIGARETTES GIVEN IN THE AFTERNOON. INDICATED TIMES ARE HOURS AFTER EXPOSURE TO LAST OF THE 4 CONSECUTIVE CIGARETTES. CONTROL ANIMALS WERE UNTREATED

[b]IND. = INDUCIBILITY

Table 7. Effect of fractions of the 1A1 cigarette-smoke-condensate on pulmonary AHH activity of C57BL/6Cum mice[a]

FRACTION NO.[b]	FRACTION	µg	UNITS/g TISSUE[c]	INDUCIBILITY
1	STARTING MATERIAL	2000	.22	1.7
2	RECONSTITUTED	500	.24	1.8
3	$B_I{}^a$	1000	.47	3.6
4	$B_I{}^b$	1000	.32	2.5
5	B_E	50	.19	1.5
6	B_W	500	.07	0.5
7	WA_I	1000	.21	1.6
8	WA_E	500	.24	1.1
9	SA_I	500	.07	0.5
10	SA_E	500	.04	0.3
11	SA_W	2000	.05	0.4
12	N_{MEOH}	2000	.32	2.5
13	N_{CH}	500	.15	1.2
14	N_{NM}	500	.43	3.3
CONTROL	CORN OIL	–	.13	1.0

[a]24 HOURS AFTER IT INSTILLATION OF FRACTION, OR CORN OIL VEHICLE

[b]ARRANGED ACCORDING TO SWAIN et al.(1969)

[c]A UNIT OF AHH ACTIVITY IS THAT AMOUNT OF ENZYME CAUSING THE FLUORESCENT EQUIVALENT OF 1 nmole 3-OH-BP/min AT 37°C

Table 8. *In vitro* effect of cigarette smoke condensate fractions on BaP metabolism in hepatic microsomes[a] from MCA treated B6 mice

FRACTION		[X]/[BaP] TO GIVE 50% INHIBITION[b]
1	STARTING MATERIAL	5.0
2	RECONSTITUTED	5.2
3	$B_I{}^a$	0.8
4	$B_I{}^b$	0.5
5	B_E	3.0
6	B_W	>10.
7	WA_I	5.0
8	WA_E	2.0
9	SA_I	>10.
10	SA_E	>10.
11	SA_W	>10.
12	N_{MEOH}	3.0
13	N_{CH}	ND
14	N_{NM}	1.0
–	7, 8-BENZOFLAVONE	1.0

[a]SOURCE OF MICROSOMES WAS HEPATIC TISSUE FROM MICE PRETREATED 24 HOURS PREVIOUS TO SACRIFICED WITH 80 µg MCA/g BODY WEIGHT GIVEN INTRAPERITONEALLY. SPECIFIC ACTIVITY OF MICROSOMES WAS 0.595 units/mg PROTEIN AND DMSO TREATED CONTROL MICROSOMES WAS 0.583 units/mg PROTEIN

[b]200, 20 and 2 µg OF THE VARIOUS FRACTIONS WERE ADDED TO THE COMPLETE REACTION MIXTURE (EXCEPT BaP), INCUBATED WITH SHAKING AT 37°C FOR 1.0 MIN., AND THEN BaP (20 µg) WAS ADDED AND INCUBATION CONTINUED FOR 20 MIN. DATA GIVEN IN TERMS OF CONCENTRATION OF BaP REQUIRED TO INHIBIT THE FORMATION OF 3-OH-BP BY 50%

concentration of the material over the concentration of BaP necessary to inhibit enzyme activity by 50% (GOUJON et al., 1973). Using a microsomal preparation from MCA-induced livers, fractions $B_I b$, $B_I a$, and N_{NM} inhibited BaP metabolism at least as effectively as the known competitive inhibitor of induced AHH, 7,8-benzoflavone. The starting material, reconstituted material, B_E, WA_I, WA_E, N_{MEOH}, and N_{CH} fractions were weak inhibitors, while the B_W, SA_I, SA_E, and SA_W fractions had no effect on BaP metabolism.

E. Discussion

There are major strain-to-strain variations in the AHH levels of inbred strains of mice (THOMAS et al., 1972). Similar variations in AHH activity (or inducibility) also exist in the human system (KELLERMANN et al., 1973a). These variations are under host genetic control in both mice and man, segregating as either a dominant, codominant, or recessive gene in mice, depending on strains employed (ROBINSON et al., 1974), and a single codominant gene in man (KELLERMANN et al., 1973b). Recent information suggests that sensitivity or susceptibility to chemically induced cancers is correlated with the AHH responsiveness of that individual. Individual mice or strains of mice which are AHH inducible are much more sensitive to MCA induced tumors than their noninducible counterparts (KOURI et al., 1973a, 1973b, 1974a); and, in man, individuals with high AHH inducibilities seem much more sensitive to cigarette smoke associated bronchogenic squamous cell carcinomas (KELLERMANN et al., 1973c). In the mouse, hepatic tissue has been used to determine the AHH inducibility of individuals. A valid question would be, does the liver activate (or inactivate) carcinogens for other tissues, or can other organs determine their own sensitivity to chemical carcinogens? Results presented here suggest that at least one other organ, the lung, can be a major determinant in its own ultimate susceptibility to chemically induced cancers.

Using the intratracheal route (to limit the enzymatic response to pulmonary tissue alone) and the B6 and D2 inbred strains of mice whose hepatic AHH responses have been extensively studied, we show in this report: 1. MCA given in a 0.2% gelatin solution induces pulmonary AHH, and this induction is dose dependent; 2. a dose of 200 µg MCA maximally induces pulmonary AHH, but has very limited effect on hepatic AHH levels; 3. pulmonary AHH can be induced in D2 mice by IT administration of MCA, but hepatic AHH levels are never induced; 4. although pulmonary AHH is induced in D2 mice, the levels are very low, with about a 5 fold difference between D2 and B6 pulmonary tissues; and 5. this strain difference is under the same genetic control as that of hepatic tissue, e.g., the highly responsive B6 strain differs from the D2 strain by a single autosomal dominant gene controlling this heightened responsiveness. Results with other inbred strains of mice agree with this contention: strains that are nonresponsive to MCA in their hepatic tissues (THOMAS et al., 1972) are low responders in their lung tissue to IT instilled MCA and vice versa.

These results may seem inconsistent in that the pulmonary tissue of both B6 and D2 mice are induced by IT administration of MCA, agreeing with the results of WIEBEL et al. (1973) and BURKI et al. (1973), yet there seem to be basic differences between these 2 "induction" processes because the responses can be discriminated genetically (Table 3). Recent results from our laboratory (KOURI et al., 1974b) suggest that there is, in fact, a difference between the "induction" of pulmonary tissues of the B6 and D2 strains. Use of the competitive

inhibitor of induced AHH, 7,8-benzoflavone, and direct quantification of the CO-binding cytochromes from B6 and D2 lung tissue demonstrate that real differences exist between the enzymes in MCA treated B6 and D2 pulmonary tissue. Available data are consistent with the hypothesis that MCA treatment of B6 lung tissue induces the genetically mediated AHH enzyme system associated with the P-448 cytochrome (SLADEK and MANNERING, 1966), and treatment of D2 lung tissue causes the nonspecific proliferation of enzymes that are very similar to the constitutive, or P-450 mediated (GILLETTE et al., 1972), enzymes. Thus, pulmonary AHH is similar, yet different, from hepatic AHH. Similar, in that AHH inducibility seems to be genetically regulated by the same locus, yet different, because there seems to be an organ specific response to MCA in even "noninducible" animals. This response may represent an adaptive response to the environment, since the lungs are constantly exposed to air and dust particles containing polycyclic aromatic hydrocarbons, insecticides, and other aromatic chemicals. This interpretation is in accord with the fact that skin is also slightly inducible in noninducible animals (WIEBEL et al., 1973).

These same strains of mice also respond by increased levels of pulmonary AHH to either whole cigarette smoke (Tables 4, 5, and 6) or particular fractions of condensates derived from this smoke (Table 7). Data are consistent with the observations of WELCH et al. (1971) and MARCOTTE and WITSCHI (1972), who showed that pulmonary AHH can be induced in rats exposed to regular or marijuana cigarettes. The lung seems to possess definite saturation levels for induction via cigarette smoke, for, regardless of schedule, only a 2 to 3 fold induction is observed. Exposure to 1, 2, or 3 cigarettes consecutively, or preexposure for up to 60 days with 8 cigarettes per day, yields quantitatively similar results. The AHH inducible C3H/fMai and B6 strains seem more responsive than the nonresponsive D2 strain (Table 5). However, after chronic exposure, both the C3H/fMai and D2 pulmonary tissues were induced (Table 6). Chronic exposure does not induce AHH levels in the liver, kidney, or intestines. Whether the pulmonary response of chronically smoked D2 mice represents true "induction" (e.g., utilizing the P-448 cytochrome) is presently being evaluated.

Results with the cigarette smoke condensate fractions (Table 7) demonstrate that the components of cigarette smoke that induce (or inhibit) pulmonary AHH can be discriminated. The relatively low inducing potential of whole cigarette smoke may reflect the presence of these inducing and inhibiting components. The BaP containing fraction, N_{NM}, is observed to be an effective inducer of pulmonary AHH and also an effective inhibitor of BaP metabolism *in vitro*. The chemical content of the potent $B_I a$ and $B_I b$ fractions is currently being determined. The phenol- or nicotine-containing fractions (WA_E and B_E) are observed to have little effect on AHH. The severe toxicity observed with the B_E fraction, however, may conceal any interaction between nicotine and AHH. Data in Table 8 nicely corroborate these IT results. Using a partially purified microsomal preparation of hepatic AHH, it was observed that certain fractions (e.g., $B_I a$, $B_I b$, and N_{NM}) are at least as inhibitory of BaP metabolism as 7,8-benzoflavone. Thus, there seems to be a correlation between ability to induce pulmonary AHH and ability to inhibit BaP metabolism *in vitro*. The most likely explanation is that these fractions contain compounds structurally similar to BaP; thus, they are capable of both inducing AHH and inhibiting BaP metabolism (competitively?) *in vitro*. The use of IT instillation of chemicals concomitant with tests for inhibition of BaP metabolism *in vitro* seems to produce rapid and reproducible tests for the detection of compounds that can potentially serve as inducers or substrates for the AHH system. Moreover, preliminary results from

the laboratory of AMES (U. of California, Berkeley) using these same smoke condensate fractions suggest that certain of these fractions (especially, $B_I a$, $B_I b$, WA_I, and neutral fractions) contain potent mutagenic activity (KIER et al., submitted, 1974). The relative carcinogenecity, and cocarcinogenecity, of these fractions are presently being tested both *in vivo* and *in vitro*.

Published results and preliminary results from our laboratory indicate pulmonary AHH may play a major role in lung cancer susceptibility. NETTESHEIM and HAMMONS (1971) reported conditions for induction of squamous cell carcinoma in inbred strains of mice. These authors utilized the (C57BL X C3H/f) F_1 and the DBA/2 strains of mice and 500 µg MCA (in 0.2% gelatin) given at weekly intervals for 4 to 6 weeks. The AHH inducible F_1 strain (KOURI, unpublished observation) was observed to be much more sensitive to MCA induced squamous cell carcinomas than the AHH "noninducible" DBA/2 strain. Very preliminary results from our laboratory involving IT administration of MCA into parent, F_1, backcross, and F2 animals (involving the B6 and D2 strains) indicate that AHH inducible mice seem to be more susceptible to MCA induced squamous cell carcinoma. Both results are compatible with the idea that the increased susceptibility to chemically induced carcinomas of AHH inducible animals reflects this heightened ability to metabolize chemical carcinogens.

F. Summary

The effects of exogenous factors on lung tissue of inbred strains of mice seem to be largely determined by the enzymatic activity of lung tissue itself. The major enzymatic activity studied in this paper was the inducible enzyme complex, AHH. It was shown that conditions could be developed so that pulmonary AHH levels were singularly effected. IT instillation of MCA (≤200 µg), exposure to whole cigarette smoke, and IT instillation of fractions of cigarette smoke condensate were shown preferentially to induce pulmonary AHH activity, and, in the case of MCA, this response was under host genetic control, segregating as a single autosomal dominant gene in crosses involving the B6 and D2 strains of mice. The small increase in D2 lung tissue following MCA treatment was attributed to nonspecific proliferation of enzymes similar to constitutive AHH, rather than a specific increase of the new P-448-mediated enzymes.

Exposure to whole cigarette smoke from either the 1A1 or 1R1 cigarette, using various exposure schedules, resulted in quantitatively similar increases in pulmonary AHH activity. Pretreatment for 60 days with 8 cigarettes per day did not increase this AHH response. Interposition of a Cambridge filter abrogated this enzyme response. Particular fractions derived from the smoke condensate of the 1A1 cigarette were observed preferentially to induce pulmonary AHH in B6 mice. These same fractions (e.g., $B_I a$, $B_I b$, N_{NM}) also were shown to inhibit BaP metabolism *in vitro*.

Results were discussed in view of the possibility that these enzymatic responses play a major role in the susceptibility of lung tissue to chemically induced cancers.

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